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The effects of multiple ancestral residues on the *Thermus thermophilus* 3-isopropylmalate dehydrogenase

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Abstract Previously, we showed that mutants of *Thermus thermophilus* 3-isopropylmalate dehydrogenase (IPMDH) each containing a residue (ancestral residue) that had been predicted to exist in a postulated common ancestor protein often have greater thermal stabilities than does the contemporary wild-type enzyme. In this study, the combined effects of multiple ancestral residues were analyzed. Two mutants, containing multiple mutations, Sup3mut (Val181Thr/Pro324Thr/Ala335Glu) and Sup4mut (Leu134Asn/Val181Thr/Pro324Thr/Ala335Glu) were constructed and show greater thermal stabilities than the wild-type and single-point mutant IPMDHs do. Most of the mutants have similar or improved catalytic efficiencies at 70 °C when compared with the wild-type IPMDH.

Keywords: Ancestral residue; Protein stability; 3-Isopropylmalate dehydrogenase; *Thermus thermophilus*

1. Introduction

Naturally occurring enzymes maintain a delicate balance between conformational stability and instability as their native structures have both stabilizing and destabilizing features. Numerous and intensive site-directed mutagenesis studies have addressed this issue [1–3]. Large changes in thermal stability often reflect the accumulation of small contributions made by many mutations. The effects of temperature on the forces contributing to protein stability are many and highly complex [4].

3-Isopropylmalate dehydrogenase (IPMDH: EC 1.1.1.85) is encoded by the *leuB* gene and is the third enzyme in the leucine biosynthetic pathway. The catalytic reaction of IPMDH is the oxidative decarboxylation of threo-D-3-isopropylmalate (threo-D-3-IPM) to 2-oxoisocaproate. The postulated evolutionary relationship between IPMDH and isocitrate dehydrogenase (ICDH) is based on their catalytically similar mechanisms and their homologous primary and tertiary structures [5,6]. These two enzymes probably originated from a common ancestral enzyme. We recently obtained many mutants containing single-residue mutations that were identified using the ancestral mutation approach [7,8]. First we inferred the common ancestral sequence of IPMDH and ICDH. Then we analyzed the effect of each ancestral residue on the thermal stability of the contemporary IPMDH from *Thermus thermophilus* (TtIPMDH) by creating mutant IPMDHs, each with one ancestral residue, and characterizing their thermal stabilities. At least six of the 12 substitutions significantly stabilize IPMDH [9]. For this study, we tested the combined effects of multiple ancestral residues on TtIPMDH stability and activity. We selected four ancestral residues (Asn134, Thr181, Thr324, and Glu335) previously found to thermally stabilize TtIPMDH [9] and, using these residues, we have now designed Sup3mut (Val181Thr/Pro324Thr/Ala335Glu) and Sup4mut (Leu134Asn/Val181Thr/Pro324Thr/Ala335Glu) as mutants with multiple ancestral residues. These mutants with multiple-point mutations are more thermally stable than are wild-type IPMDH and mutants with single-point mutations.

2. Materials

3-Isopropylmalate and NAD^+ were purchased from Wako Pure Chemicals and Orient Yeast Ltd., respectively (Tokyo, Japan). All other reagents were analytical grade. Water was purified using a Milli-Q purification system (Millipore, Tokyo, Japan).

3. Construction of multiple-point mutants

The genes for Sup3mut (Val181Thr/Pro324Thr/Ala335Glu) and Sup4mut (Leu134Asn/Val181Thr/Pro324Thr/Ala335Glu) were obtained by PCR-based site-directed mutagenesis [10]. To construct Sup3mut, a plasmid harboring the Pro324Thr mutant gene was used as the template [9]. The following oligonucleotide primers were used for the mutagenesis: 5′-CTTGTCCACGCT**CGT**CACGTGCTTCCTG-3′ (Val181Thr mutation) and 5′-AGCGCGGGCACGGAG**GAG**TTCACGGCCACGGTC-3′ (Ala335Glu mutation). To construct Sup4mut, the Sup3mut gene was used as the template. The oligonucleotide primer 5′-TAGATCCCCCCGGT**GTT**CTCCCGGACGATGAG-3′ (Leu134Asn mutation) was used for mutagenesis. For the primer sequences given above, mutated codons are shown in bold type. T7P and T7T primers flanked the cloned genes in the vector pET21c (Novagen, Tokyo, Japan) and were used to amplify the DNA fragments harboring the complete coding sequence. The amplified DNA fragments were digested with *Nde*I and *Bam*HI and then were cloned between the same restriction sites into pET21c. The DNA sequences of the mutant *leuB* genes were confirmed using the dideoxy chain termination method, a

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Abbreviations: TtIPMDH, 3-isopropylmalate dehydrogenase from *Thermus thermophilus*; ICDH, isocitrate dehydrogenase; IPM, *threo*-DL-isopropylmalate

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Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Tokyo, Japan), and a 3100 DNA sequencer (Applied Biosystems).

4. Expression and purification of multiple-point mutant IPMDHs

The wild-type and mutant IPMDHs were purified as described by Hayashi and Oshima [11]. In short, the wild-type and mutant *leuB* genes were over expressed in *Escherichia coli* MA153 grown in 2 X YT medium (16 g trypton, 10 g yeast extract, and 5 g NaCl per liter) supplemented with ampicillin (50 μg/ml). The cultures were incubated at 37 °C until the A_{600} reached 0.7. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Expression continued for three hours at 37 °C. The *E. coli* cells were harvested by centrifugation and stored at −80 °C. Cell pellets were suspended in 20 mM potassium phosphate, pH 7.6, 0.5 mM EDTA and then sonicated. The cell extracts were held at 75 °C for 20 min and then centrifuged to remove insoluble material. The enzymes were purified using DEAE-cellulose (DE-52; Whatman, Maidstone, England), Butyl-Toyopearl 650S (Toso, Tokyo, Japan), and Resource Q (GE Healthcare Bio-Sciences, Tokyo, Japan) columns. The purities of the recombinant enzymes were confirmed by SDS–polyacrylamide gel electrophoresis. The purified enzymes were stored in the presence of 60% saturated ammonium sulfate at 4 °C. The quantity of protein was estimated using a BCA Protein Assay Reagent Kit (Pierce, Rockford, USA) and bovine serum albumin as the standard.

5. Assay method

IPMDH activity was monitored using a spectrophotometric method [11]. The standard reaction buffer contained 50 mM 2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, 0.4 mM *threo*-DL-isopropylmalate, and 5 mM NAD^+. The change in absorbance at 340 nm, which parallels NADH production, was recorded and used to calculate the initial rate. The kinetic parameters, K_m and k_{cat} for NAD^+, were determined by measuring the initial rates of the catalytic reactions with a saturated concentration of 0.4 mM IPM and 0.03–1 mM NAD^+ at 70 °C. The K_m values for IPM were determined with a saturated concentration of 5 mM NAD^+ and 0.01–0.2 mM IPM at 70 °C.

6. Analysis of thermal stability

The IPMDHs were dissolved in 20 mM potassium phosphate buffer, pH 7.6, 0.5 mM EDTA and dialyzed against the same buffer overnight. Enzyme solutions were then incubated at various temperatures for 10 min or at 86 °C for different periods of time. The samples were cooled on ice and then centrifuged (21 000 × *g*, 20 min) to remove insoluble material. The activity remaining in each supernatant was estimated under standard conditions at 50 °C. Though the activity is higher at higher temperature (70 °C), results are more reproducible at lower temperature (50 °C). Accordingly, the remaining activity was routinely estimated at 50 °C.

7. Thermal denaturation estimated by far UV CD measurement

Thermal denaturation of the IPMDHs was monitored by recording their circular dichroism (CD) at 222 nm with a Jasco-J-720 Spectropolarimeter (Jasco International Co., Tokyo, Japan). A 0.1 cm path-length quartz cell was used. The solution temperature was increased at a rate of 0.25 °C/min using the programmable temperature controller PTC-348WI (Jasco International Co., Tokyo, Japan).

8. Result and discussion

We previously inferred the ancestral sequence of IPMDH and ICDH using sequence alignment and phylogenetic tree construction [8,9]. Fig. 1 shows the sequence alignment of IPMDHs, ICDHs, and the postulated ancestral enzyme.

In the previous study [9], we have found seven ancestral residues that stabilize IPMDH: Thr16Val, Pro56Glu, Leu134Asn, Val181Thr, Asp184His, Pro324Thr, and Ala335Glu. However, Pro56Glu mutant was less stable than the wild-type IPMDH when the remaining activity was compared, thus the residue was not considered. Among the six other stabilizing ancestral residues, spatially well-separated four residues were selected: Leu134Asn, Val181Thr, Pro324Thr, and Ala335Glu.

From the structural point of view, it is not obvious why each ancestral residue improves the stability of IPMDH [9]. Leu134 is located near the active site residues Leu90, Leu91, Arg104,

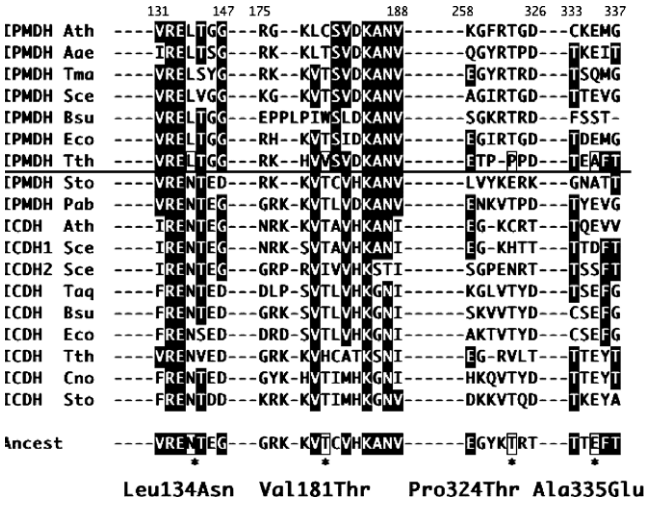

Fig. 1. Multiple alignment of IPMDHs and ICDHs from selected organisms. Four sequential regions (residues 131–147, 175–188, 320–326, and 333–337 of *T. thermophilus* IPMDH) are shown. The bottom sequence is the ancestral sequence predicted by the maximum likelihood method [8,9]. Asterisks (*) indicate ancestral residues that were incorporated into Sup3mut and/or Sup4mut. Abbreviations for the IPMDHs and ICDHs, with the accession numbers given in parentheses, are: IPMDHAth and ICDHAth, *Arabidopsis thaliana* (AAD21684, AAM91080); IPMDHAae, *Aquifex aeolicus* (NP213167); IPMDHTma,*Thermotoga maritima* (NP228366); IPMDHSce, ICDH1 Sce, and ICDH2Sce, *Saccharomyces cerevisiae* (NP009911, NP014361, NP014779); IPMDHBsu and ICDH Bsu, *Bacillus subtilis* (NP390705, NP390791); IPMDHEco and ICDHEco, *Escherichia coli* (NP414615, NP415654); IPMDHTth and ICDHTth, *Thermus thermophilus* (AAA16706, P33197); IPMDHSul, *Sulfolobus tokodaii* (NP376313); IPMDHPab,*Pyrococcus abyssi* (NP127027); ICDHTaq, *Thermus aquaticus* (AAB39334); ICDHCno, *Caldococcus noboribetus* (T44658); ICDHSul, *Sulfolobus tokodaii* (NP378162).

Tyr139 and Arg194. It is expected to be difficult to introduce mutations near the active site without affecting activity. The Leu134Asn mutant was the most stable mutant. However, we were unable to determine why the Asn134 mutation stabilizes IPMDH. The threonine residue replacing Val181 may contribute to an increase in hydrogen bonding in this region of the protein, although we are unable to predict its hydrogen bonding partner. Pro324 is located in a loop that contains three proline residues (Pro323, Pro324 and Pro325). This region has a special structure and is concerned with thermal stability in IPMDH. In the previous study [9], Pro324Thr mutant showed an unexpected increase in thermal stability. We suspect that a potential hydrogen bond may have formed between Thr324 and main-chain carboxyl group of the main chain of Ala331 located 0.449 nm from the site of the mutation. Ala335 is located within an α-helix and is facing the molecular surface, although the residue at position 335 is buried. The increase in the stability of this mutant may be due to ion pair formation between Glu335 and Arg342, which are located approximately 0.45 nm apart.

To test the combined effects of the ancestral residues, mutants containing multiple-point mutations were constructed. The sequences of such mutant *leuB* genes, constructed by site-directed mutagenesis, were verified by DNA sequencing. We constructed Sup3mut (Val181Thr/Pro324Thr/Ala335Glu) and Sup4mut (Leu134Asn/Val181Thr/Pro324Thr/Ala335Glu) genes. The mutants were then expressed in *E. coli* and purified separately.

Fig. 2 shows the residual activity estimated at 50 °C after heat treatment for 10 min at various temperatures ranging from 75 to 95 °C for each IPMDH. The associated $T_{1/2}$ value is the temperature at which half of the enzymatic activity is lost by the end of the treatment. The $T_{1/2}$ values for Sup3mut and Sup4mut are higher than that of the wild-type enzyme. The $T_{1/2}$ for Sup3mut is 3.4 °C higher and the $T_{1/2}$ for Sup4mut is 5.5 °C higher than that of the wild-type enzyme. The $T_{1/2}$ values are listed in Table 1.

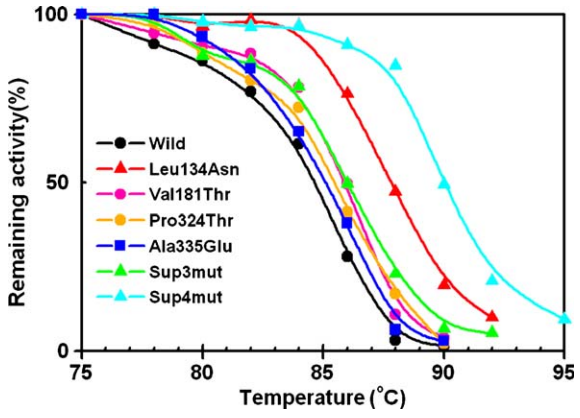

Fig. 2. Thermal inactivation of wild-type (TtIPMDH) and its mutants. Each purified enzyme was diluted to 10.9 μM with 20 mM potassium phosphate buffer, pH 7.6, 0.5 mM EDTA and treated at the temperatures indicated for 10 min. Remaining activity was estimated at 50 °C under standard assay conditions. Symbols: black circles, wild-type; red triangles, Leu134Asn; pink circles, Val181Thr; orange circles, Pro324Thr; blue squares, Ala335Glu; green triangles, Sup3mut (Val181Thr/Pro324Thr/Ala335Glu); sky blue triangles, Sup4mut (Leu134Asn/Val181THhr/Pro324Thr/Ala335Glu).

Table 1
Thermal stabilities of the wild-type and mutants of TtIPMDH

Protein	Remaining activity		CD measurement
	$T_{1/2}$ (°C)[a]	$t_{1/2}$ (min)[b]	T_{CD} (°C)[c]
Wild-type[g]	84.7 (0.0)[d]	5.6 (1.0)[e]	87.4 (0.0)[f]
Leu134Asn[g]	87.9 (+3.2)	16.7 (3.0)	91.3 (+3.9)
Val181Thr[g]	86.1 (+1.4)	10.5 (1.9)	89.1 (+1.7)
Pro324Thr[g]	85.4 (+0.7)	7.9 (1.4)	87.7 (+0.3)
Ala335Glu[g]	85.1 (+0.4)	7.4 (1.3)	88.3 (+0.9)
Sup3mut (Val181Thr/Pro324Thr/Ala335Glu)			
	88.1 (+3.4)	13.8 (2.5)	90.9 (+3.5)
Sup4mut (Leu134Asn/Val181Thr/Pro324Thr/Ala335Glu)			
	90.2 (+5.5)	150 (27)	92.7 (+5.3)

[a] $T_{1/2}$: apparent half-denaturation temperature as estimated from Fig. 2.
[b] $t_{1/2}$: apparent half-denaturation time when treated at 86 °C as estimated from Fig. 3. The $t_{1/2}$ value for Sup4mut was estimated from the data collected after prolonged incubation up to 3 h at 86 °C.
[c] T_{CD}: apparent half-denaturation temperature as estimated from the denaturation profile monitored by CD at 222 nm (Fig. 4). The standard error of the mean is ±0.1 °C and is derived from triplicate denaturation experiments using wild-type IPMDH.
[d–f] The figures in parentheses indicate relative values normalized to the value for the wild-type enzyme (e) or the difference from the value of the wild-type enzyme (d and f).
[g] Values are from Ref. [9].

The thermal inactivation time courses for mutant IPMDHs that were heated at 86 °C are shown in Fig. 3. The half-denaturation time, $t_{1/2}$, for Sup3 is 2.5-fold greater than that of the wild-type enzyme. The $t_{1/2}$ value for Sup4mut was estimated from the data collected after prolonged incubation up to 3 h at 86 °C and is 27-fold grater than that of the wild-type enzyme (Table 1).

To monitor secondary structure denaturation induced by temperature, CD spectra were recorded. The far-UV CD-spectra of native Sup3mut and Sup4mut are indistinguishable from that of the wild-type enzyme at room temperature (data not shown). Fig. 4 shows the thermal denaturation profiles of the wild-type enzyme, the single-point mutants, and the multiple-point mutants when monitored by measuring their 222 nm CD values. T_{CD} values, i.e. the temperatures at which half of the proteins' secondary structures are lost, were estimated from the data of Fig. 4 and are listed in Table 1. The T_{CD} value

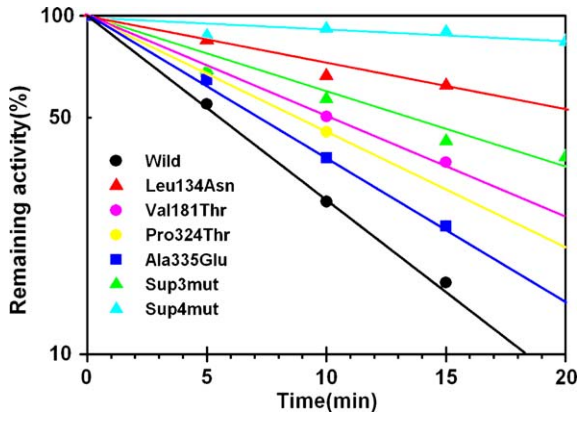

Fig. 3. Thermal inactivation time courses for wild-type TtIPMDH and its mutants. Each purified enzyme was diluted to 10.9 μM with 20 mM potassium phosphate, pH 7.6, 0.5 mM EDTA and treated at 86 °C for the times indicated. Symbols are the same as in Fig. 2.

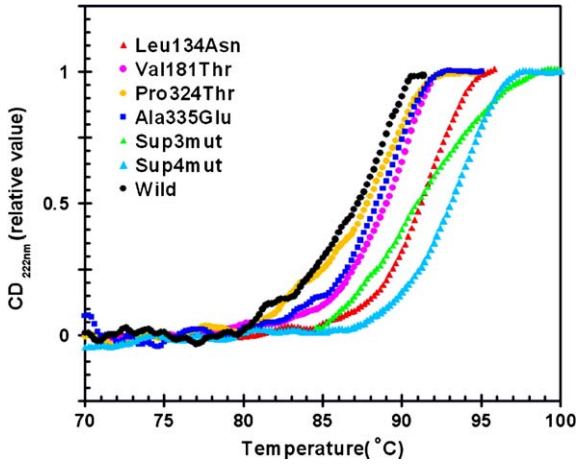

Fig. 4. Denaturation curves for wild-type TtIPMDH and its mutants as estimated by CD at 222 nm. Protein samples (5.4 μM) were dissolved in 20 mM phosphate, pH 7.6. The temperature was increased at a rate of 0.25 °C/min. Symbols are the same as in Fig. 2.

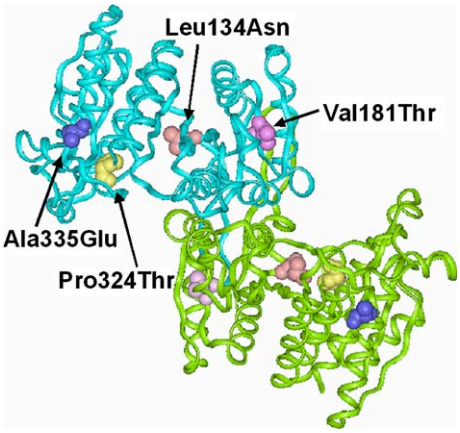

Fig. 5. Three-dimensional structure of the TtIPMDH dimer with one subunit shown in sky-blue and the other in green. The residues substituted with ancestral residues in this work are indicated. The figure is drawn using the Insight II program.

for Sup3mut is 3.5 °C higher and the T_{CD} value for Sup4mut is 5.3 °C higher than that of the wild-type enzyme. Sup3mut and Sup4mut have greater thermal stabilities than does wild-type TtIPMDH. Sup4mut is also more stable than is any single-point mutant.

Table 2 summarizes the kinetic parameters for wild-type and mutant IPMDHs at 70 °C. Seven of the single-point mutant enzymes, Phe53Leu, Pro56Glu, Arg58Leu, Leu134Asn, His179Lys, Pro324Thr, and Ala335Glu, have improved K_m values for NAD at 70 °C. The k_{cat} values for nine single-point mutants, Phe53Leu, Pro56Glu, Arg58Leu, Val61Ile, Leu134Asn, His179Lys, Ser261Asn, Pro324Thr, and Ala335Glu, are larger than that of wild-type IPMDH. For nine of the eleven single-point mutants, their catalytic efficiencies (k_{cat}/K_mNAD) are improved at 70 °C. The K_m for NAD and k_{cat} values found for Sup3mut are similar to those of the wild-type enzyme. However, Sup4mut shows improved K_mNAD and k_{cat} values. The k_{cat}/K_mNAD ratio for Sup4mut is improved by 3.81-fold. Because K_m values for IPM are very low at 70 °C and are difficult to estimate accurately, the K_m values were measured only for the three most stable mutants. The K_m values for IPM for Leu134Asn and Sup3mut are similar to that of the wild-type enzyme. Though the K_mIPM value of Sup4mut is higher than the wild-type, the $k_{cat}/(K_m\text{NAD} \cdot K_m\text{IPM})$ is 1.4-fold of that of the wild-type IPMDH. These results suggest that the inclusion of ancestral residues often bolsters the catalytic efficiency at 70 °C.

A cumulative effect is generally observed when combining stabilizing and/or destabilizing mutations [12,13]. This property indicates that the residue changes induce only local structural perturbations and that they introduce (un)favorable interactions that (de)stabilize the protein structures independently against denaturation. Fig. 5 shows the three-dimensional structure of the TtIPMDH dimer and the ancestral residues introduced in this experiment. The mutations, Leu134Asn, Val181Thr, Pro324Thr and Ala335Glu, are well separated spatially. Incorporation of multiple ancestral residues into TtIPMDH results in enhanced thermal stability without negatively affecting catalytic efficiency. This study is the first examination of the effects of multiple ancestral residues on stability.

Table 2
Kinetic parameters of enzymatic reactions of ancestral mutants and TtIPMDH

Enzyme	Stability[a]	70 °C			
		K_mNAD (μM)	K_mIPM (μM)[b]	k_{cat}NAD (s^{-1})	k_{cat}/K_mNAD (s^{-1} μM^{-1})
Wild-type	87.4 (0.0)	514 ± 16.9 (1.00)	6.1 ± 0.5 (1.00)	13.4 ± 0.2 (1.00)	0.026 (1.00)
Phe53Leu	86.0 (−1.4)	468 ± 17.4 (0.91)		15.0 ± 0.9 (1.12)	0.032 (1.23)
Pro56Glu	88.7 (+1.3)	428 ± 23.3 (0.83)		14.6 ± 0.6 (1.09)	0.034 (1.31)
Arg58Leu	84.0 (−3.4)	401 ± 7.0 (0.78)		17.7 ± 0.4 (1.32)	0.044 (1.69)
Val61Ile	87.2 (−0.2)	520 ± 21.9 (1.01)		15.0 ± 0.4 (1.12)	0.029 (1.12)
Leu134Asn	91.3 (+3.9)	172 ± 4.5 (0.33)	7.0 ± 1.4 (1.10)	29.9 ± 0.3 (2.23)	0.174 (6.69)
His179Lys	87.2 (−0.3)	336 ± 5.5 (0.65)		21.3 ± 0.3 (1.59)	0.063 (2.17)
Val181Thr	89.1 (+1.7)	642 ± 3.7 (1.25)		10.2 ± 0.2 (0.76)	0.016 (0.62)
Asp184His	88.8 (+1.4)	673 ± 10.0 (1.31)		11.2 ± 0.2 (0.84)	0.017 (0.65)
Ser261Asn	85.6 (−1.8)	625 ± 11.4 (1.22)		18.2 ± 0.5 (1.36)	0.029 (1.12)
Pro324Thr	87.7 (+0.3)	371 ± 15.6 (0.72)		13.8 ± 2.2 (1.03)	0.037 (1.42)
Ala335Glu	88.3 (+0.9)	359 ± 24.4 (0.70)		18.2 ± 1.5 (1.36)	0.051 (1.96)
Sup3mut	90.9 (+3.5)	567 ± 45.7 (1.10)	6.3 ± 1.5 (1.03)	13.2 ± 1.0 (0.99)	0.023 (0.88)
Sup4mut	92.7 (+5.3)	255 ± 27.4 (0.50)	16.1 ± 3.5 (2.64)	25.2 ± 2.4 (1.88)	0.099 (3.81)

[a] T_{CD} is the apparent half-denaturation temperature estimated from the denaturation profile monitored by CD at 222 nm. The standard error of the mean is ±0.1 °C and is derived from triplicate denaturation experiments using wild-type IPMDH.
[b] K_m values for IPM were estimated only for three most stable mutants.

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